INHIBITION OF THE PHOSPHORYLASE KINASE CATALYZED REACTION BY GLUCOSE-6-P*

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SUMMARY

Kinetic studies show that glucose-6-P inhibits the phosphorylase kinase reaction. With the use of alternative substrates, a tetradecapeptide and phosphorylase b covalently modified at the allosteric site, no inhibition was observed. These observations show that glucose-6-P inhibits the phosphorylase kinase reaction by its effect on the substrate, phosphorylase b, and that binding an activator counteracts the effect of the inhibitor. Inhibition by glucose-6-P depends upon Mg^{++}, and kinetic studies showed that inhibition is competitive with respect to phosphorylase b and mixed with respect to ATP.

INTRODUCTION

The control of the synthesis and degradation of glycogen is of central importance in the energy metabolism of skeletal muscle. Regulation is influenced by glucose-6-P because it is an allosteric activator of glycogen synthase (1) and an allosteric inhibitor of glycogen phosphorylase b (2). Glucose-6-P can influence both synthesis and degradation of glycogen by its effect on enzymic interconversion; e.g., dephosphorylation of glycogen synthase D (3) and phosphorylase a (4) are both stimulated by it. Krebs et al. (5) showed that glucose-6-P is an inhibitor of phosphorylase kinase. The mode of this inhibition, however, was not characterized. In this work, we examine

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the kinetics of inhibition of this reaction and provide evidence that the action of the carbohydrate is due to binding with the substrate, phosphorylase b.

MATERIALS AND METHODS

Crystalline rabbit-muscle phosphorylase b was prepared according to a procedure of Fischer and Krebs (6). ^{32}P-labeled phosphorylase a was prepared by using ^{32}P-ATP (7) with purified phosphorylase kinase (8). ^{32}P-tetradecapeptide was isolated after chymotryptic digestion of ^{32}P-phosphorylase a by a method of Nolan et al. (9). A dephosphorylated tetradecapeptide was obtained by using ^{32}P-tetradecapepetide with purified bacterial alkaline phosphatase as described by Tessmer and Graves (10). Phosphorylase b modified at the AMP site was prepared by reacting enzyme with 8-[m(m-fluorosulfonylbenzamido)-benzylthio]adenine (11).

Phosphorylation of the peptide by phosphorylase kinase was measured by an assay as described by Tessmer and Graves (10). The conversion of phosphorylase b to a was measured by the incorporation of ^{32}P from ^{32}P-ATP (12) or by measurement of enzyme activity (8).

RESULTS AND DISCUSSION

The phosphorylase kinase reaction is inhibited by glucose-6-P when the conversion of phosphorylase b to a is measured either by the change of enzymic activity or by the incorporation of ^{32}P from ^{32}P-ATP (Table I). Inhibition depends upon the level of glucose-6-P and occurs approximately to the same extent at pH 6.8 and 8.6. The results presented in Table I could be explained by the action of glucose-6-P on the protein substrate, phosphorylase b, the enzyme, or both. To distinguish between these possibilities, we used a small molecular-weight alternative substrate, a tetradecapeptide (10). It was reasoned that this peptide would have little affinity for glucose-6-P and that, if inhibition was substrate directed, binding to phosphorylase b, no inhibition would be found with the peptide

Table I. Effect of Glucose-6-P on Interconversion of Phosphorylase b to a

Additions	% Enzymic Activity		% Phosphorylation	
	pH 6.8	pH 8.6	pH 6.8	pH 8.6
None	100	100	100	100
5 mM Glucose-6-P	72	79	---	---
10 mM Glucose-6-P	54	48	48	57

Reaction mixtures containing inactive kinase (1.1 μg/ml) and 3.6 mM ATP (or $AT^{32}P$), 12.5 mM Mg^{++}, 25 mM glycerol-P, 25 mM Tris, 1 x 10^{-4} M phosphorylase b, and varying amounts of glucose-6-P at 30° C. Initial velocity was measured by phosphorylase a formation (8) or ^{32}P incorporation (12). Activity of phosphorylase kinase in the absence of glucose-6-P is taken as 100%.

Table II. Effect of Mg^{++} on Glucose-6-P Inhibition of Phosphorylase Kinase

Mg^{++} (mM)	% Inhibition
8	46
16	31
32	25
48	23

Reaction mixtures containing 0.8 μg/ml kinase, 2.4 mM ATP, 25 mM glycerol-P, 25 mM Tris, 6 x 10^{-6} M phosphorylase b and varying amounts of $Mg(AC)_2$, at pH 8.6. Phosphorylase kinase activity in the absence of 10 mM glucose-6-P is taken as 100% activity.

substrate. With the use of about 0.5 mM peptide at pH 8.6, no inhibition of phosphorylation by glucose-6-P could be detected. Thus, it seems that inhibition of the phosphorylase kinase reaction with phosphorylase b is due to the binding of glucose-6-P on the substrate, phosphorylase b.

AMP is known to counteract the inhibition of phosphorylase b by glucose-6-P (13). Also, it has been demonstrated that AMP antagonizes activation of phosphorylase phosphatase by glucose-6-P (14). Therefore, it was thought important to know whether AMP could overcome inhibition of phosphorylase kinase by glucose-6-P. At 0.1 mM, AMP had little or no effect on phosphorylase kinase activity, which agrees with earlier results of Krebs et al. (5). AMP at 1 mM caused some inhibition of phosphorylase kinase, but AMP at 0.1 mM or 1 mM was unable to abolish inhibition by glucose-6-P. The lack of effect by AMP could be due to the conditions of the test system; e.g., ATP that is present at 3.6 mM could bind to phosphorylase b and block the action of AMP. To simplify the interpretation of the data, an alternative substrate was used again. For this case, we used phosphorylase b modified at its allosteric site with 8-[m(m-fluorosulfonylbenzamido)benzylthio]adenine (11). This enzyme forms shows similar properties to those obtained with phosphorylase b and AMP (11), including a lack of sensitivity to glucose-6-P. With this enzyme derivative, glucose-6-P (10 mM) caused no inhibition of the phosphorylase kinase reaction. We conclude that binding an activator to the allosteric site can block glucose-6-P inhibition and that the failure of AMP to overcome the inhibition probably is related to a competition between the nucleotides, AMP and ATP, for phosphorylase b in the assay.

The activity of phosphorylase b is influenced by Mg^{++}; e.g., an increase in Mg^{++} concentration causes an increase in affinity of phosphorylase b for AMP (15). Also, inhibition of phosphorylase b by glucose-6-P decreases with an elevation of Mg^{++} (16). Because the effect of glucose-6-P on phosphorylase kinase also is due to the binding to phosphorylase b, it might be expected

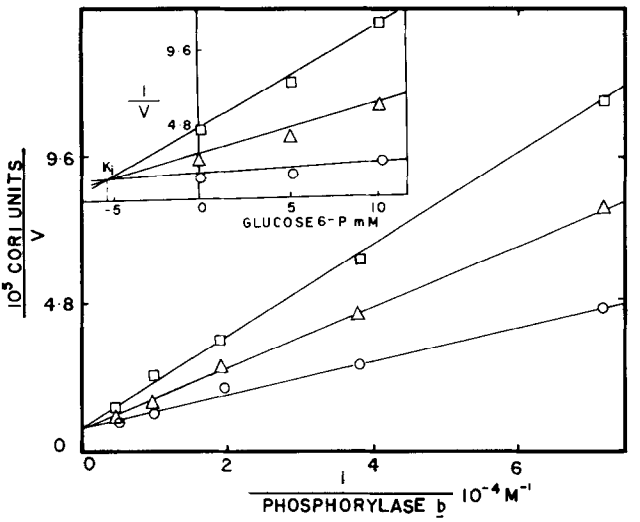

Figure 1. Double reciprocal plot for phosphorylase b in the presence of glucose-6-P. The reaction mixtures contained inactive phosphorylase kinase (1 µg/ml), 12.5 mM Mg^{++}, 3.6 mM ATP, 50 mM glycerol-P, 50 mM Tris, pH 8.6 and varying amounts of phosphorylase b. (○) No additions; (△) 5 mM, and (□) 10 mM glucose-6-P. Initial velocity was measured by phosphorylase a formation. Insert: Dixon plot ($\frac{1}{v}$ vs. glucose-6-P) for the inhibition of phosphorylase kinase by glucose-6-P at the following concentrations of phosphorylase b; (○) 5 x 10^{-5} M, (△) 2.5 x 10^{-5} M, and (□) 1.4 x 10^{-5} M.

that Mg^{++} would influence the inhibition of phosphorylase kinase reaction. Table II shows that as Mg^{++} is increased over the ATP concentration (2.4 mM), the inhibition decreases. Our data suggest that the effect of Mg^{++}, at least in part, is due to its influence on the substrate, phosphorylase b.

Kinetic studies were undertaken to examine the nature of inhibition of phosphorylase kinase by glucose-6-P. In the presence of glucose-6-P, phosphorylase kinase exhibits a decreased affinity toward the substrate, phosphorylase b, without changing the V_{max} (Fig. 1). Thus, it seems that glucose-6-P is a competitive inhibitor with respect to phosphorylase b. A K_I of 6.5 mM was determined. The insert shows that the $\frac{1}{v}$ vs. glucose-6-P plot consists of a family of straight lines. Competitive kinetics can be explained by the formation of a substrate-inhibitor complex that is poorly recognized by phosphorylase kinase. Two mechanisms are possible: (I) The phosphorylase b-glucose-6-P complex is not bound by phosphorylase kinase. Inhibition is reduced by raising the concentration of phosphorylase b. At

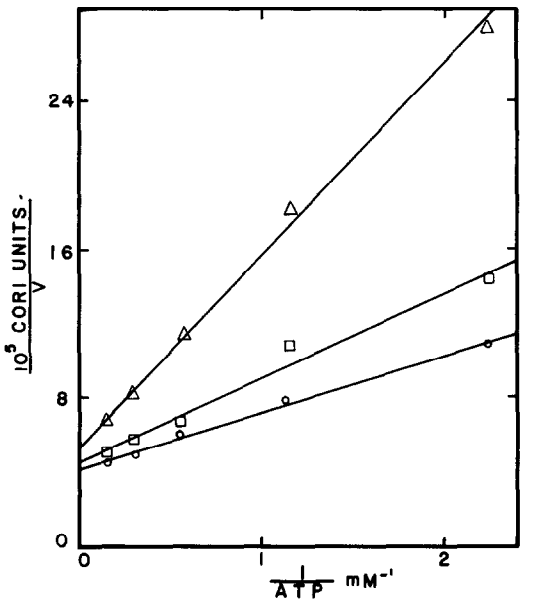

Figure 2. Double reciprocal plot for ATP in the presence of glucose-6-P. The reaction mixtures containing inactive phosphorylase kinase (1 μg/ml), 12.5 mM Mg^{++}, 5.85 x 10^{-5} M phosphorylase b, 50 mM glycerol-P, 50 mM Tris, pH 8.6 and varying amounts of ATP. (○) No additions, (△) 10 mM, and (□) 20 mM glucose-6-P. Initial velocity was measured as described in Fig. 1.

infinite phosphorylase b, the level of the substrate-inhibitor complex is negligible to free phosphorylase b, and inhibition is nil. (II) The phosphorylase b-glucose-6-P complex is poorly bound to phosphorylase kinase in comparison with free phosphorylase b, but both species are phosphorylated with identical V_{max} values. The kinetics of glucose-6-P inhibition with respect to ATP also was examined. Figure 2 shows that the inhibition is of a mixed type; e.g., both V_{max} and the enzyme affinity toward ATP are affected by glucose-6-P.

Inhibition of phosphorylase kinase by glucose-6-P can occur at the level of glucose-6-P found in skeletal muscle. Upon electrical stimulation of skeletal muscle, contraction ensues, glycogenolysis occurs, and glycolytic intermediates increase; e.g., after 20 sec of tetanic contraction, glucose-6-P increases from its rest value of 0.3 mM to 3.6 mM (17). After a 10-sec tetanus, it seems that the muscles become fatigued (17), and phosphorylase a

levels decline rapidly (17,18,19). Activation of phosphorylase phosphatase by glucose-6-P probably contributes to the decrease in phosphorylase a activity (4,13). Our in vitro experiments with the inhibition of the phosphorylase kinase reaction by glucose-6-P suggest that this mechanism also may be important in controlling the level of phosphorylase a in fatigued muscle.

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